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Researches on the formation of diastase by *Aspergillus niger* van Tieghem

by

G. L. FUNKE

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Researches on the formation of diastase by *Aspergillus niger* van Tieghem

by

G. L. Funke.

I. CRITICAL SUMMARY OF LITERATURE.

Many investigators have already considered the question, whether bacteria and fungi are able to modify the secretion of their enzymes qualitatively and (or) quantitatively under the influence of nutrition. Most of them have however arranged their experiments in a very primitive way, in that they were not careful to exclude all possible disturbing influences, and almost all of them tackled the subject from a different angle. It does not therefore, surprise us in the least that very contradictory results have been obtained.

Even the first investigators of this question are unable to agree, whether the organisms only form their enzymes in the case of necessity i. e. whether the secretion is influenced qualitatively by nutrition. Wortmann e. g. stated in 1882 that bacteria only secrete amylase when cultivated on starch. As he however worked with a mixture of putrefying bacteria, even he himself could not name the material, used for his experiments.

Büsgen however mentions in 1885 that *Aspergillus Oryzae* produces amylase even when glucose is the only available source of carbon.

Fermi 1890—1891 cultivated bacteria on proteids and on sugars, and found that in the case of proteids only proteolytic enzymes are formed. In 1895 however he found with Montesano that *Aspergillus niger* secretes invertase, when glycerine is given as the only source

of organic foodmaterial. Pfeffer on the other hand, in the same year, expressed as his opinion that as a rule, organisms accomodate their enzymes to the foodmaterial, which is present. If *Aspergillus niger* is cultivated on a mixture of starch and glucose, it will secrete amylase only then, when the glucose has been consumed and the only foodmaterial, available for the fungus, is starch.

In the laboratory of Pfeffer, and also in the experiments of Katz, who made a more detailed investigation in 1898, the fungi (*Aspergillus niger* and *Penicillium glaucum*) were cultivated on several kinds of sugar, always with the addition of starch, usually 0.25%. He observed, that the presence of the sugars diminishes the production of amylase, and that this decrease is direct proportional to their concentration. In the case of *Penicillium* even a low sugar-concentration is enough to stop the secretion of amylase entirely. He finds that diastase is produced, even when glucose is the only organic food present. This was the first time that a quantitative modification of enzyme production had been observed.

It is rather a pity that Katz was satisfied with investigating from day to day whether there was any starch left in the culture liquid; if the iodine reaction was negative, the experiment was not continued. A single observation of the culture on glucose was enough for him to draw his conclusions.

Duclaux as early as 1899 remarked that *Aspergillus niger* and *Penicillium glaucum* produce many enzymes, but that nutrition could be said to influence their secretion only within very wide limits. Went in 1901 came to similar conclusions. He found ten enzymes in the case of *Monilia sitophila*. These he divided into three groups according to the influence that nutrition had on their secretion: one group was always present, no matter what culture media were used; another group which was not formed in all media, but in several of them, and a third group of specific enzymes, which were only formed when the materials acted upon by these, were supplied in the culture medium of the fungus.

Butkewitsch too observed, that foodmaterials had some influence on the quantity of the enzymes produced. As, however,

he only used two different kinds of nutrient bases, we must be cautious in accepting the conclusions he drew from his results.

Pottevin in 1902 investigated the secretion of lactase by *Aspergillus niger*, and he found that lactase was only secreted when the specific reaction of that enzyme could take place.

Dox thinks, after his investigations on *Penicillium camemberti* in 1910, that all the enzymes, which an organism is capable of producing, are secreted at all times; that therefore nutrition only has a quantitative influence on the secretion of enzymes and that the more of a given enzyme is needed, the more is secreted. Colin 1911 and Grezes 1912 are of the same opinion.

In 1911 Kylin investigated this matter more fully, but he did not succeed in throwing any further light on the subject. He cultivated fungi on several substances, always with the addition of starch, and from day to day tested whether any starch was left in the culture solution. The more sugar there was in the solution, the longer the starch took to disappear. As soon as this happened, he considered the experiment as ended. He found that diastase production took place even in culture solutions without starch, and always found this the case in the many different foodmaterials he used. In all these cases diastase was formed; when however starch was added to these culture solutions, more diastase was produced, and still more when starch was the only available foodmaterial. Kylin considers this to be a very good instance of the quantitative influence of nutrition on enzyme secretion.

All these researches have a common source of error: no attention was paid to the age of the fungi in question. Yet we can scarcely imagine that a fungus will secrete the same quantity of enzyme throughout its whole lifetime; on the contrary, it is possible that a certain quantity is formed and later on disappears for some, as yet, unknown reason. In any case, we are not entitled to compare the results of two experiments in which organisms of different ages have been used. These theoretical considerations caused Went in 1914 to investigate the secretion of diastase by *Aspergillus niger* during its whole lifetime. As a culture solution he used a 5% solu-

tion of glucose, containing the usual inorganic salts. Every day, later on every second or third day he tested whether there was any diastase present in the mycelium and in the culture liquid, and if diastase was found, he determined the concentration of the enzyme. In this way he was able to determine the influence of the age of the organism on the secretion of the enzyme, and to find at what point this secretion is at a maximum.

If the same is done with culture solutions of different composition to Went's, we may be able to collect material from which some valuable conclusions may be drawn. This was the object of the following research.

I have only quoted the literature in broad outlines; under the various subdivisions of this subject I shall always refer to the articles concerned with that part of it.

II. METHOD OF INVESTIGATION AND SOURCES OF ERROR.

A. Method of investigation.

The method of doing this type of research has been fully described by Went. As my method differed only very sligthly from his, a brief description should suffice.

The following salts and concentrations were used in all the culture solutions

NH_4NO_3	0.5 %
K_2HPO_4	0.1 %
$MgSO_4$	0.05 %

These remained unaltered; as organic foodmaterials carbohydrates chiefly were added to this solution. Their concentration and composition will be given as each experiment is discussed. 75 c.c. of this solution were poured into a flask, and after being sterilised, they were all inoculated with the fungus at the same time. 40 à 50 flasks formed one series. The solutions were inoculated in the following way: a quantity of conidia was brought into a test tube

containing sterilised water, and consequently they spread over the surface. With a looped platinum wire a drop of liquid was taken from the surface and dropped into one of the flasks. One must admit, that in this way we introduce a more or less equal number of conidia into each vessel. There was always an equal development in all the flasks; if there was a difference, it was only visible during the first few days and was very slight.

The cultures were kept in a room in which the temperature was kept constant by automatically regulated electric heaters at 22° (later 20°). Oscillations of more than 0.2 were very rare. By keeping flat open zinc basins filled with water in the room the humidity of the air was also kept constant. As the room had no windows, the cultures were in the dark, except during the times, when the observations were made. I never found any influence of the electric light on the cultures. There were no gaspipes in the room.

Germination as a rule had proceeded far enough after two or three days, to enable me to begin the experiments. During the first week two cultures were taken every day and during the second week every other day. After that, cultures were only taken every third or fourth day. The cultures were treated as follows: the culture solutions were filtered off. In one case the mycelium was filtered through a dried and weighed filterpaper and was dried in a dessiccator afterwards until the weight was constant. In this way we are able to find accurately how much fungus had been developed.

The other mycelium was thoroughly washed to remove all the adhering culture solution and diastase. It was then rubbed down with infusorial earth and extracted with culture solution which had been boiled, so as to destroy all the possible enzymes in it. Sometimes 75 c.c. water were used for the extraction. After standing an hour, the mixture was filtered and the filtrate was tested for diastase. The culture solution of the first jar was also tested in the same way. Often a third culture was used and the mean of the results taken.

To test the concentration of the diastase, the following procedure

was adopted: I made a solution of potato starch which had been prepared by Lintner's method; 1 Gr. of this starch was dissolved in 1250 c.c. water (therefore a solution of 0.08%). The water was constantly stirred to prevent boiling. A quantity of this solution, as as rule 25 c.c., was mixed with an equal volume of the liquid, which had to be examined; the time at which this took place was noted. From time to time a small portion of this mixture was taken away, after thoroughly shaking the liquid. This portion was tested for the presence of starch and erythrodextrine by the addition of a little dilute solution of J in JK[1]. When the liquid remained yellow without the slightest touch of red, I took it for granted that all the starch was hydrolysed. Of course it is rather difficult to find the exact time, taken for complete hydrolysis, but it can best be determined by taking the mean of the time taken by the last test showing a faint redish-yellow tint and the first test that remains yellow. I was always very careful to have these last two observations as close to each other as possible. In most cases the time, taken for hydrolysis could be expressed in minutes, some of the shortest only took seconds to be complete.

Went divided the mixture of mycelium extract or culture solution and starch equally amongst a large number of test tubes. It has however been observed that the distribution of the enzyme in the liquid is not always quite homogeneous. In this way it has happened that hydrolysis proceeded faster in one test tube than in another. For this reason it is my imperative to shake the liquid thoroughly before taking a portion of it for testing. Besides pipetting an equal amount of the solution into a large number of tubes only lengthens the experiment unnessecarily.

Making use of the time taken for hydrolysis, I estimated the concentration of diastase in the same way as Went. I took the amount of diastase to be = 100 if the time, taken for hydrolysis under the above conditions was 150 min. If for instance in a given

[1] Although there were sometimes small differences in the concentration of the iodine. I never remarked, any influence of it.

case the time taken is 54 min., then the quantity of enzyme will be $\frac{150}{54} \times 100 = 277.8$.

Besides the dry weight of the mycelium and the amount of diastase, I also tested the degree of acidity of the culture solution and of the mycelium extract regularly, and I sometimes also tested how much sugar was left in the culture solution.

What remained of the different solutions was carefully stored. These were used on the following days to see how long the liquid retained its fermentative action. I shall return to these in detail later on.

For the sake of completeness I may mention, that the influence of alien organisms is excluded by adding a few drops of toluol to the liquids.

The main objection to this method is in my opinion, that by testing with iodine, the time of hydrolysis can not be determined with a sufficient degree of accuracy. Of course testing it by means of rotation or by power of reduction would be much more accurate, but very difficult because of the low concentration of starch used for the experiment. Besides, for my subject, very great accuracy has no practical value, because I only use the results to compare them with others in a long table, the sumtotal of the columns of which give me an indication of the course, followed by the enzyme secretion. As we shall see later, I have been rather successful in obtaining a clear idea of this, even though my conclusions are based on the observations of more than one culture. For unluckily we are forced to deal with a large number of cultures in which individual differences are unavoidable. I have reason therefor to believe that this lack of accuracy can not be regarded as such a serious drawback, that it would prevent us from knowing exactly, how the secretion of the enzyme goes on during the lifetime of the fungus.

A very great advantage of this method is, that it takes so little time. It was only because of this, that I have been able to investigate about a thousand different cultures. I need not point out that

many drawbacks are vitiated by the fact I was able to collect such a vast mass of material. It is better to find the same broad outlines in a large number of cases, than to base your conclusion on a single observation, although that may be very accurate.

B. Sources of errors.

There are many sources of error in a research of this type. We can never avoid them entirely and I believe that in this work we shall never be able to obtain any absolute results, but the values we find will only be approximately and relatively exact. It must be understood that I interpret the word source of error in a very wide sense. I also include under this term the inherent variability of the organism itself. This variability is so great and influenced so much by facts, which are very easily neglected, that I feel sure that, so far as I know, there are not two investigations on *Aspergillus niger*, which can be compared with each other.

Not only does the chemical composition of glass exert a great influence on the habitus, formation of conidia and physiological qualities of the fungus, but there may be very important differences with the same type of glass, when new, and after it has been used for some time, as Hanna Lappalainen has conclusively shown. I worked with globe shaped flasks with straight necks and after using them to cultivate fungi a few times, I replaced them by new ones of the same kind. This glass splits off alkalies when water is boiled in it and also when used to sterilise the culture solution. If 75 c.c. water were boiled in a flask for a quarter of an hour, phenolphtaleïne gave a distinct red colour. One drop of 0.1 N acid was sufficient to neutralise it again. To find, whether new vessels differed amongst themselves and from those that had been used for some time, I boiled some water in them and then titrated with weak acid of 0.005 N. I obtained the following results: 50 c.c. of water, boiled in a new flask, were neutralised by 10.9, 34.1 and 12.7 c.c. of acid respectively; the same quantity in the case of used vessels was neutralised by 33.3 and 23.1 c.c. of acid. There were therefor very great differences, but we are not entitled to

assume that old flasks always split off more alkali than new ones.

The flasks were sterilised by heating them on three consecutive days to 100° for half an hour. The solutions of reducing sugars (glucose, maltose and lactose), which at first were colourless, became light brown. I see the cause of this in the presence of the alkali: probably it caused an enolisation, also giving rise to ketones, according to the following equation

$$\underset{\text{aldose}}{R.CHOH.CHO} \rightleftarrows \underset{\text{enol}}{R.COH.CHOH} \rightleftarrows \underset{\text{ketone}}{R.CO.CH_2OH} \qquad \text{(Boëseken)}$$

We must therefore remember, that in the series of cultures on reducing sugars, some of the sugar is always changed into other substances. *Aspergillus niger* forms enough acid on the first days to neutralise all the alkali; I therefore paid no more attention to it. Of course it would have been better to use Jena glass, but as there were always 200—300 flasks in use, the available supply in the laboratory would not have been sufficient.

Hanna Lappalainen informs us too that there are cultures of *Aspergillus niger* from all parts of Europe in the laboratory of professor Elfving in Helsingfors. Amongst these, their seem to be eight variaties, which differ physiologically from each other. Brenner claims to have discovered three new ones. He compares them to the mutations, which have been described by Schiemann, but in my opinion, he is not entitled to do so. Schiemann obtained them by adding poisonous salts to the nutrient bases, while Brenner obtained his by cultivating his fungi at different temperatures for a very long time. The fungi are naturally influenced to a very large extent, but if we allow them to grow in their original environment long enough, they again assume their original qualities. In this way he himself succeeded in getting back an old race out of a mutation.

The investigations of Lappalainen and Brenner were made with a totally different object in view than mine. They observed and described different physiological reactions and I am unable to say which of the different races of the fungus, described by them, I

used. I can only say that I got my stock from a pure culture, which I obtained from the Phytopathological Laboratory "Willie Commelin Scholten" in Baarn (Holland); from this stock my first cultures were made.

One of the main reasons, why Brenner succeeded in getting so many races, is the high temperature, at which he grew his fungi, i. e. 35°. At this high temperature many complications appear, which I never noticed when working at 22° and 20°, e. g. the formation of starch in the hyphae. Boas also has warned us, that *Aspergillus niger* shows many pecularities when cultivated at high temperatures.

In this connection I may mention, that there is another consideration of very great importance, namely with which conidia a given series is inoculated. I sometimes took two series of experiments and inoculated one of them with conidia from a culture, in which the same nutritive liquid was used, whilst the second series was inoculated with conidia from a different culture solution. In all such cases great differences were observed between the two series: dry weight, diastase production, habitus, formation of conidia, all these points differed in fungi, derived from conidia of different origin. As yet I have not found any fixed rules for these changes, but I hope to return to this question farther on. I am however able to point out some very remarkable instances now. The tables I A and I B give the results of an investigation of diastase formation by two series, both of which were cultivated on a 5% glucose solution. The conidia of the first series were taken from a culture on 5% glucose, I therefore call this series G G. The other series was inoculated with conidia from a culture on 0.5% starch, hence A G. Fig. 10 gives the reader an idea of the production of diastase in two series, the first of which was grown on 0.5% starch with conidia from a culture solution of the same composition (A A), the second also on 0.5% starch, but the conidia came from a culture solution containing 5% glucose (G A).

In all these cases the conidia were derived from well developed cultures, which were never more than three or four weeks old. We

can not therefore imagine that the phenomenon observed, is due to a lessened power to germinate.

Up to now it was not thought that the influence of a former culture medium could be so great and as a result, this fact was never considered in experiments. Yet we certainly must do so and we should not describe any differences as a new race before a thorough investigation. Only Grezes has, as far as I know, observed a similar phenomenon in *Aspergillus niger*. He found a quantitative difference in the secretion of invertase where the conidia had been derived from cultures on saccharose or succinic acid. But in his case there had been at least sixty generations in tbe same culture solution before and in my experiments I have seen, that it is not necessary at all that the same culture medium should be used for a long time before any marked difference can be observed. I found that growing a fungus for two generations on the same culture solution was quite sufficient. It is certainly worth while to compare these facts with the observations of miss Westerdijk and van Luyk, who obtained similar results, when working with the spores of *Gloeosporium*.

It is very difficult to decide on a definite line of conduct amongst all these uncertainties; I decided to inoculate all my series with conidia which had been grown on culture solutions of the same composition. Whenever I have deviated from this course, special mention will be made of it. I think that any possible investigators of this question in the future should adopt the same procedure, because only if they do this, they will have the right to compare their results with mine.

The very striking difference between my first results and those of Went, made me consider whether possibly the size of the exposed surface of the culture solution could influence the physiological behaviour of the fungus. Went worked with a surface of 24, later 48 squ.c.M., I with 47 squ.c.M. It did nct seem very probable that this could cause variations. Yet I performed an experiment; although I did not find the cause for the differences mentioned above, it was not wholly without results. 75 c.c. of

culture solution containing 1% glucose were put into Erlenmeyer flasks of two different sizes, six of each. The surface in the small flasks was 26 squ.c.M. in the big flasks 82 squ.c.M. They were inoculated on the same day; observations were made on the 8th 14th and 21st days. The results can be seen from the following table:

days	diastase in the myc.	P_H of the myc. extract	diastase in the cult. solution	P_H of the cult. sol.	dry weight in m.Gr.
		26 squ.c.M.			
8	93.5	3.6	158	3.57	168
14	277.8	3.2	1200	3.1	282
21	187.5	3.5	2000	3.35	251
		82 squ.c.M.			
8	300	3.2	320	3.1	342
14	214	3.2	1500	3.1	296
21	100	3.4	1788	3.3	237

From this table we see, that during the first week the secretion of enzyme, the dry weight of the mycelium, as well the degree of acidity are less in the cultures with the smaller exposed surface. After the first week or so however, these things are equalised.

The investigations made during the last twelve years on the influence of the hydrogen ion concentration on the action of enzymes, forced me to find, how the diastase of *Aspergillus niger* reacts to the acidity of the culture medium. I was sure from the beginning that it could not be without any influence at all, because *Aspergillus niger* produces very much acid and the amylase it secretes must therefore work at a high hydrogen ion concentration.

This investigation was made in the way, first described by Sörensen. It is based on the use of standard solutions, the hydrogen ion concentration of which is known. These standard solutions and the liquids, the P_H of which we wish to determine are coloured by indicators, so that we can determine the turning point exactly by comparing the colours. The standard solutions I used,

were prepared according to the receipts of Clark and Lubs. The pure chemicals I needed, were kindly supplied to me by Dr. I. M. Kolthoff, while prof. Dr. W. E. Ringer personally tested the strength of the solutions by electrolysis. I wish to thank these two gentlemen whose valuable help enabled me to carry out this part of my investigation without encountering too many difficulties.

The experiment itself was very simple. A certain quantity of enzyme solution (10, 15 or 20 c.c.) was mixed with an equal volume of a 0.16 or 0.32% starch solution. To obtain various degrees of acidity, I always added the standard solutions themselves in volumes, equal to those of the enzyme and starch solutions. As they are buffer solutions at the same time, $H^{\cdot}$ was constant throughout the experiment. The P_H of the mixture was determined and also the time, taken for the hydrolysis at the various degrees of acidity. For further particulars I refer the reader to the articles of Sörensen and Michaëlis.

The results are plotted in the usual way in fig. 1. This same

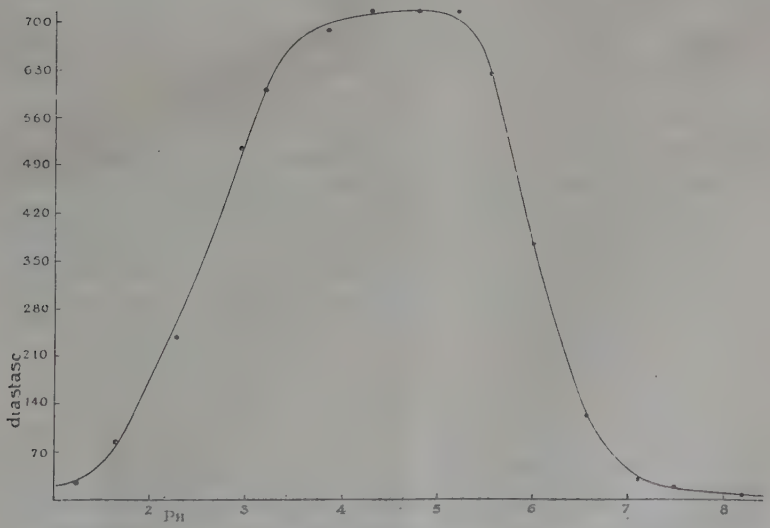

Fig. 1. Influence of the hydrogen ion concentration on the action of the diastase of *Aspergillus niger*.

curve was found in all my experiments, both for the culture solution as for the mycelium, no matter what the concentration of the diastase was. The amylase of *Aspergillus niger* seems to be influenced very slightly by the degree of acidity i. e. the optimal acidity for the enzyme has very wide limits. It is fortunate, that the fungus itself keeps the acid concentration of the culture solution optimal, so that we do not need to acidity the liquids artificially[1]. Sometimes even the acidity may be too high, but then during the experiment the liquid is mixed with neutral starch solution and by this means the P_H is again brought down to the optimum.

In the above experiment great accuracy was imperative. From fig. 1 it can be seen that this is possible by using iodine as an indicator. I may also mention that there is a close agreement between my results and those of Adler on the influence of the culture medium on the diastase of malt.

It is clear therefore that in this experiment I need only consider the acidity in the following case. From the curve we see, that $P_H = 6$ is already less than optimal, at $P_H = 7$ the action is almost zero. The mycelium itself contains hardly any acid. When extracted with water, the solution has a P_H of $\pm = 6$ and this

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[1] It is not selfevident at all that the fungus should produce the optimum hydrogen ion concentration itself. This can be seen from an investigation I made on the diastase concentration of cultures of *Monilia sitophila*. These cultures were three weeks old and were grown on 5% lactose. The culture solution had a $P_H \pm = 8$, and the hydrolysis of starch proceeded very slowly. When however the degree of acidity was artificially increased, hydrolysis was much faster. The following results were obtained

$P_H = 3.95$	hydrolysis in 52 minutes
5.40	31
5.90	32
± 6.50	36
± 7.50 (natural state)	500

This result is only a preliminary investigation, but it shows that in all these experiments we should be careful to know the optimum P_H at which the enzyme must be observed.

sinks to $P_H + = 6.5$ when starch solution is added. In this case therefore, the diastase does not work under optimal conditions and the values we obtain for its concentration are too low. Unfortunately I had already noted a number of observations from experiments, performed in this way before I was aware of the mistakes I made. I did so in order to avoid all possible influences of admixtures in the culture solution. If however the results are influenced at all by this, it must be very slightly. The curve in fig. 1 is very gradual. This would not have been the case, if the result had been profoundly influenced by those admixtures; otherwise we might have expected at the very least, that there should be obvious irregularities in the curve.

From the above it is obvious that we meet with many difficulties in a research of this type. How far I have been able to overcome these may be evident in the following chapters.

III. RESULTS AND CONCLUSIONS, DRAWN FROM THE SERIES CULTIVATED ON GLUCOSE, STARCH AND MALTOSE.

A. Results.

I have put the results I obtained for the above series into tabular form. Before proceeding to discuss each one of these series separately, I wish to make a few general remarks which apply to nearly all of them. As an exemple I may take table 3, which shows the results of the series that was cultivated on a solution containing 2.5% glucose. From the headlines of the columns we can see to what the figures refer.

Between the 13th and 16th day all the food has been consumed, as can be seen from the rotation caused in the polarimeter by the culture solution (2^{d} col.). The maximum dry weight is reached some time before that (9th col.). So even before assimilation has ceased, dissimilatory processes have already gained the upper-hand. This is found to be the case in most series; only sometimes

the two processes coincide. After the maximum dry weight has been reached, the weight of the fungus begins to diminish, first comparatively rapidly, later on very slowly. Sometimes it may even remain constant for several days.

The degree of acidity of the culture solution (8th col.) also rises gradually until about the 13th day, then it sinks for some time and after that it remains constant. The same naturally is true of the mycelium extract (6th col.), if it has been extracted with the boiled culture solution. Very often the hydrogen ion concentration rises above the optimum, but I have already mentioned why this is no drawback to the experiment (see page 232). We see however that this is certainly the case for the extract which is got by extracting the rubbed down mycelium with water. In this case the P_H is always too low (4th col.).

That this is really the case, can be seen by comparing the values, got of diastase concentration that were obtained at this low degree of acidity (3d col.) and the values from the extract with the culture fluid, where the P_H is optimal (see also tables 7—9; 2nd and 3d col.; the P_H has not been reported there, but it does not differ from the analogous ones in table 3).

It is worth while to compare the production of diastase in the mycelium with that in the culture solution. In both cases we find a strong production up to about the 7th day. After that the diastase concentration of the culture solution falls only to rise again to a much higher level. The production of diastase in the mycelium shows no regular rise or fall, but only great oscillations. Something like it can be seen in the tables no. 2, 4, 8 and 9, for resp. 4% glucose, 1% glucose, 0.25% starch + 2.5% glucose and 0.4 % starch + 1% glucose.

In all these cases, the diastase concentration in the mycelium, compared to that of the culture solution, is so small, that it is almost negligible. My own impression is, that the fungus secretes its diastase into the surrounding medium and that the diastase, found in the mycelium, must be regarded as having remained there more or less accidentally.

Indeed we can see that this quantity is always very variable and dependent on accidental circumstances from the following table. This represents the quantity of diastase, formed in a short series, which was cultivated on a solution, containing 0.5% starch. In this experiment I did not boil the culture solution before using it to extract the mycelium with it. In this way I have arranged side by side the enzyme values for mycelium + culture solution and for culture solution only. If the enzyme in the mycelium really were an independent and important value, we should expect to see the values in the first column of the table to be appreciably higher than those in the second. We see however that in the beginning the latter values are only slightly smaller, later on they are even higher. On the 4th and 5th days only there seem to be appreciable quantities in the mycelium, but after that practically all the enzyme is secreted into the culture solution.

days	diastase in myc. + cult. sol.	diastase in the cult. sol. only	dry weight in m.Gr.
3	0.75	0	17
4	84.5	3.45	35
5	93.8	6.8	50
6	90.5	75.—	56
7	90.3	160.8	74
8	214.9	225.—	96
9	177.25	312.—	130
10	266.8	410.7	118

(these results are the averages of the observations, made on at least two cultures.)

Fig. 2 (next page) represents these values graphically. We are struck by the fact, how much more regular the diastase production is in the culture solution only (·····), than the production in the mycelium and the liquid together (———).

If at the same time we turn up tables 7—9, col. 2 and 3, we are struck by the fact that not the least regularity can be detected in the development of enzyme in the mycelium, whether we extract it with water or with the boiled culture solution. We naturally

expect that the extract with water should always show much lower values than the extract with the culture solutions. This is by no means always the case; the former is often even higher than the latter. Again the diastase values in the culture solutions agree very well. There may be important variations on the same day, but the general course of events in both is the same. These facts support my view that the quantity of diastase in the mycelium is only accidental.

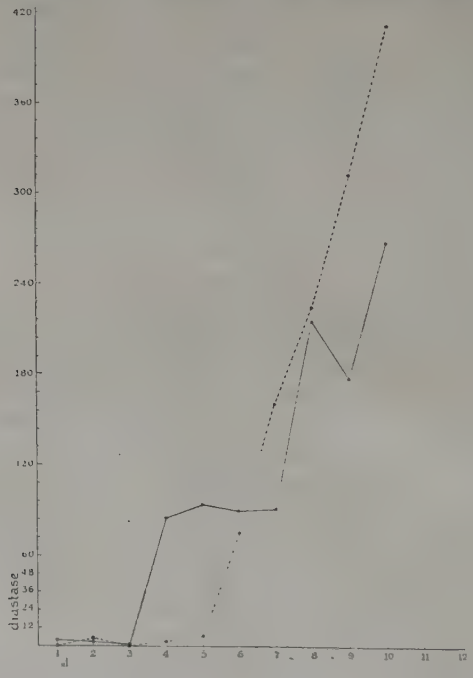

Fig. 2. Diastase in mycelium + culture solution (————); diastase in the culture solution only (--------), (d = days)l

The enzyme is secreted in the first stages of active development, so that I can not favour the view that the diastase in the liquid is only derived from cells, which have died and allowed the enzyme to pass out. I feel sure that the diastase has passed to the outside through the living protoplasma.

In discussing the series, not much needs to be said about the degree of acidity. I should only like to briefly consider what acid it is, that causes the high hydrogen ion concentration, especially as my results do not tally with those of earlier investigators. Benecke states that *Aspergillus niger* produces oxalic acid, when nitrogen is supplied in the form of an organic compound. If ammonia salts are given however, only the NH_4 ion is assimilated, while NO_3 ion forms nitric acid, which prevents the formation of organic acids. Wehmer confirms this statement and adds, that the acid does not check the growth of the fungus, but checks the development of conidia. After some time the acid should disappear, because the basic substances, formed by the breaking down of proteids, combine with it. We shall see that these two last assertions are only partly true.

Boas and Leberle traced the acidity from day to day and they found the most development of acid when NH_4NO_3 was given. They also found, that the acid did not decrease as was the case, when organic nitrogen compounds were given. They accounted for the decrease by assuming, that the fungus itself assimilated the oxalic acid, which was first produced.

The results of Elfving agree very well with the others. He also finds citric acid besides oxalic acid. As however the nutritive value of the former is very high, it is consumed much more rapidly than the latter.

It is a pity, that none of these investigators extended their investigations over more than ten or twelve days. They have a very exaggerated idea of the influence of the acid on the fungus and they think that the acid disappears sooner or later in all cases. They are strengthened in their erroneous belief by the fact that the degree of acidity gradually sinks during the last few days of their observations. If only they had proceeded with the experiments for a few more days, they would have seen their mistake. This is seen very clearly in all my tables, where the P_H has been determined. Besides I have been unable to find even a trace of organic acid, when NH_4NO_3 was used as the nitrogenous foodmaterial.

There certainly seems to be a relation between the amount of fungus-matter and the amount of acid formed. When the fungus was poorly nourished, i. e. on 0.5%—2.5%, the P_H was not so high (2.9), as when a solution, containing 4%—5% was used (1.8).

After this I need not crave your attention again for the dry weight of the mycelium. Its behaviour is clearly seen in table 3, and is very much the same in all cases. The exact time at which the maximum dry weight was reached, was more or less the same always. The numerical value of the maximum weight however, differs very much and was directly proportional to the amount of foodmaterial supplied, so far as glucose and starch are concerned. With the strongest solutions, 5%, about 23% was changed into fungous material; in the weakest solutions, 0.5%, 31% was used in the same way. Between these two extremes, there is a regular gradation of the percentages of the foodmaterial, assimilated by the fungus. This can clearly be seen from fig. 3.

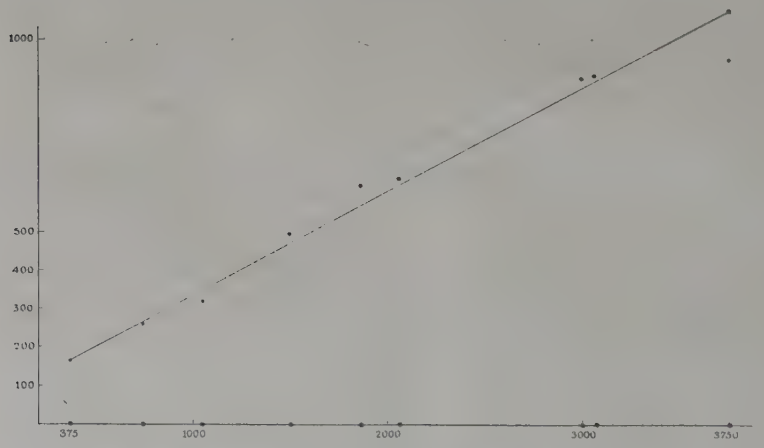

Fig. 3. Maximum dryweight of the mycelium at different concentrations of foodmaterial; this too has been expressed in m.Gr.

To save the reader much unnecessary looking up of tables, I will add in brackets on what days the maximum weight was reached and its numerical value in milligrams i. g. glucose 4% (13—16;

912). It is always imperative to know on what days the maximum weight was reached as it is the key to maximum development. Pfeffer obviously did not think of this (l. c. page 257, note). He only determined the weights of the fungi between the 20th and the 26th days. In this way his figures can not be compared with each other, and his results must necessarily be of little value.

In the following pages our chief interest will be the amount of enzyme that is formed. To give a more vivid impression of my results, I plotted most of them on squared paper and so obtained a number of instructive curves. In most cases I estimated the values, found for the culture solution alone, to give a clear enough impression of the production of diastase by the fungus; so I neglected the amount of enzyme in the mycelium. In some cases I added both amounts to plot them into a curve. I found that the main points in these curves were influenced only very slightly by this procedure.

The values I found do not seem to lend themselves readily to graphical representations. Although the daily oscillations are not big enough to disturb the general outline and scheme of the curves, yet they are so great, that they disturb very much their regularity. Many of these irregularities may be the results of errors of observation, others are due to individual differences of the cultures. Especially cases, where only short times are needed for complete hydrolysis are very troublesome to observe accurately. If for example we find the times for complete hydrolysis expressed in minutes to be: 6—6.5, 5—8—7.5, 5—6—8.5 etc. then the enzyme values, corresponding to these figures are: 2500—2222—1875—2143—2000—1765. These numbers would on a graph cause great irregularities. Yet it is clear that we are dealing here with values, which are more or less of the same order. I feel justified therefore to use a reducing factor which enables me to accentuate the essential differences of these values and at the same time cuts out accidental and unimportant variations. As a very convenient factor I chose to take the logarithms of all these numbers; instead of the large ones given above, we then get the following series: 3.38—3.35

—3.27—3.33—3.30—3.38—3.25, which gives a more satisfactory representation of the course of events. So in studifying the graphs, it must be born in mind that the quantities of diastase have not been represented by the actual numbers, but by their logarithms. If we were to look for actual enzyme values on the curves, confusions would be sure to arise.

A still simpler way of graphic representation of the results would be to plot the times, taken for hydrolysis from above downwards on the ordinate (see fig. 8). There is however one great drawback to this method: if e. g. the time taken for hydrolysis in a series of days were the following: 2000—600—110—35—12—4—3—3.5 min. etc. (similar values are often obtained in the beginning of the experiments), we should have to cook either the highest or the smallest numbers to allow them to come out in the graph at all, and it is doubtful whether we could do this successfully. When however it was possible to use this method, I did so and it will be seen that in the main points the curve is the same as when I plotted the logarithms.

Glucose 5% (11—19; 944.5) fig. 4, table 1 A.

GG: i. e. inoculated with conidia from a culture on glucose 5%

Glucose 5% (9—16; 1217) table 1 B.

AG: i. e. inoculated with conidia from a culture on starch 0.5%.

It is very certainly important to compare the tables, obtained by grouping the results of the above two series. The aftereffect of the former culture medium on the conidia, which were used for the

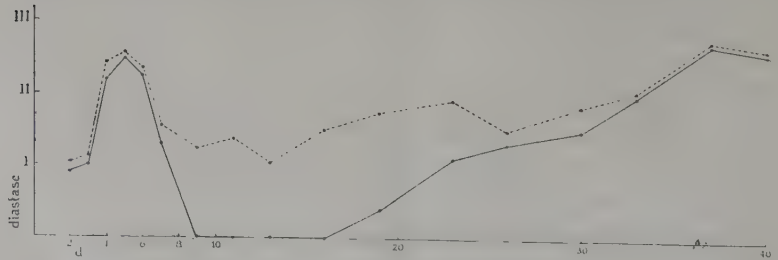

Fig. 4. Glucose 5%. Diastase in the mycelium + culture solution (--------); diastase in the culture solution alone (———); (d = days).

inoculation, is very evident. The dry weight in the series AG is strikingly higher than in the other. Hardly any conidia were formed in this series and if so, it was only along the sides of the vessel; in the centre the mycelium remained white. In the GG series however, the mycelium was black throughout.

Very little diastase is produced in AG, so that I did not think it worth while to represent it graphically. In the case of GG however, this production is of importance. We see a fairly considerable rise in both curves, viz. for diastase in mycelium + culture solution (———) and also in the culture solution only (--------). Before the maximum dry weight is reached, both curves descend. On about the 9th day, the minimum of enzyme is found; in the culture solution it may even go down to zero. Later however there is another rise, which is even greater than that in the beginning.

Glucose 4% (13—16; 912), fig. 5, table 2.

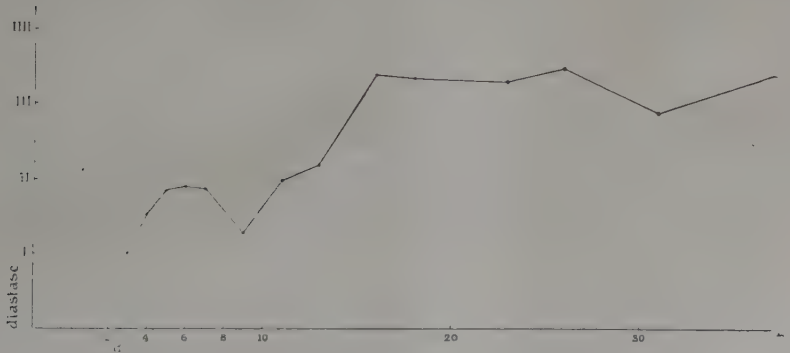

Fig. 5. Glucose 4%. Diastase in the culture solution, (d = days).

In this case also abundant conidia were formed, so that soon the mycelium was black throughout. This is true of all cultures on glucose and starch. The mycelium always formed a flat, continuous pellicle. Undulations in it were only found in the very thickest of them.

The production of diastase is very similar to that of glucose 5%. The first maximum is not so high and the subsequent fall is not so

great. The lowest point is reached on the 9th day. The second rise begins earlier and rises to a higher level than with glucose 5%.

Glucose 2.5%. (11—13; 615.5) fig. 6, table 3.

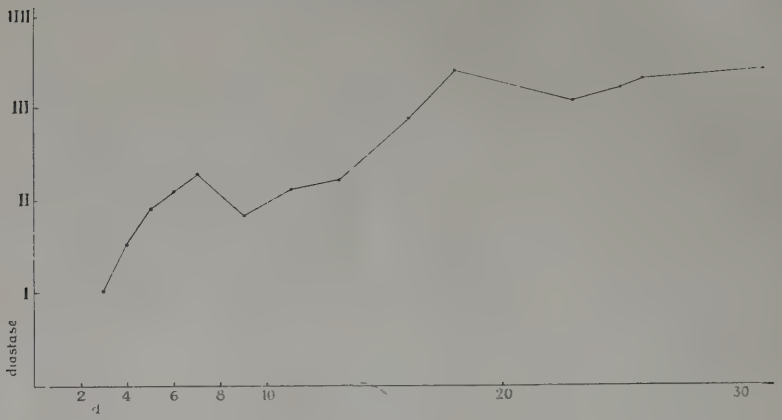

Fig. 6. Glucose 2.5%. Diastase in the culture solution. (d = days).

Here again the results are very similar to the above series, but the differences seen between the 5% and the 4% solutions are still more marked here. The minimum, as shown by the lowest point in the curve, is reached on the 9th day.

Glucose 1%. (9—16; 261) fig. 7, table 4.

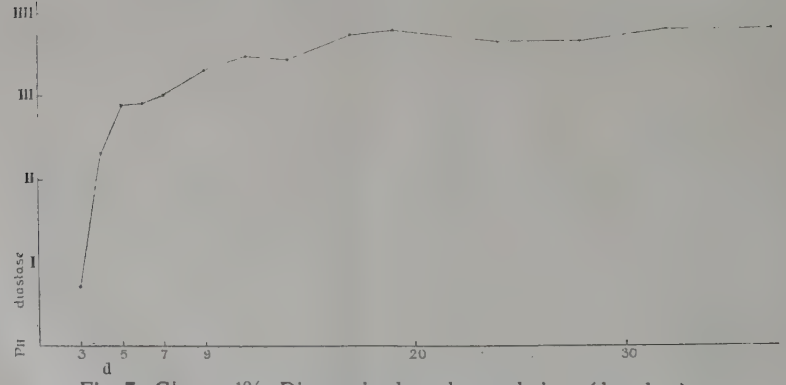

Fig. 7. Glucose 1%. Diastase in the culture solution. (d = days).

In this case there is only just an indication of a fall in the curve, but there is a much stronger secretion of enzyme than has been observed in any of the other cases.

Fig. 8 shows the course, taken by the secretion of diastase in the last three series plotted according to the second method, as

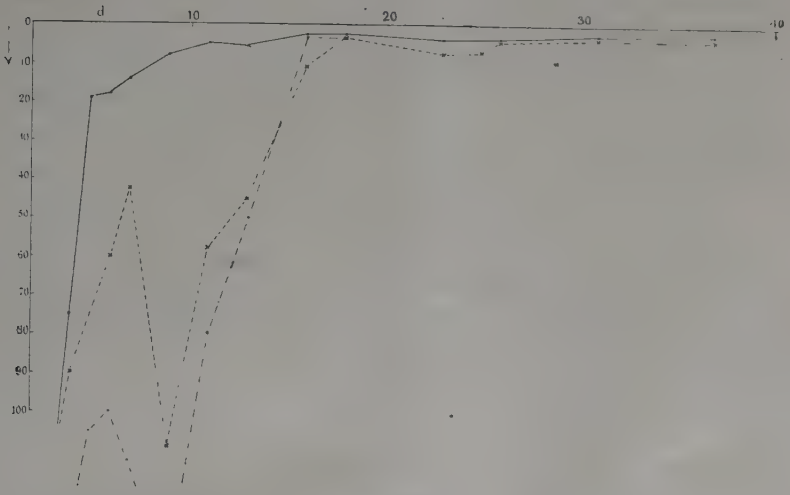

Fig. 8. Glucose 1% ———; glucose 2.5% ·····; glucose 4% —·—·— (omzettingstijden = times taken for hydrolysis; d = days); (for the last days the lines have only been drawn partly, in order to avoid indistinctnesses in the figure).

described on page 240. It is unnecessary to comment on the curve as the course of events is evident enough from it.

Turning to the tables on the series containing starch, the reader will find the day on which the iodine reaction is negative for the first time, marked by a cross. If we turn to the cultures on mixtures of glucose and starch, we find that my results do not agree with those of Kylin. It is also very obvious, that the experiment begins to be really important after all the starch has disappeared from the solution.

As long as there was starch present in the solution, the enzyme concentration was determined as follows. Two equal volumes of

the culture solution were mixed, one with an equal volume of water and the other with an equal volume of 0.08% starch solution. In this way the H' in both cases was equal. The mixture to which starch had been added, took a longer time to give a negative iodine test. This difference of time was taken to be a measure of the amount of diastase present in the original solution. It is an open question, whether this method is quite accurate. As however it is only used during the first few days of development, during which there are no important events in the secretion of diastase, I thought that there is very little to be said against using this method.

Starch 2%. (11—22; 493) fig. 9, table 5.

During the sterilisation of the culture solutions, a small precipitate of starch came down. This was weighed in a few cases; the average weight of the sediments was found to be about 8% of the total amount of starch used. This therefore can not influence the experiment materially.

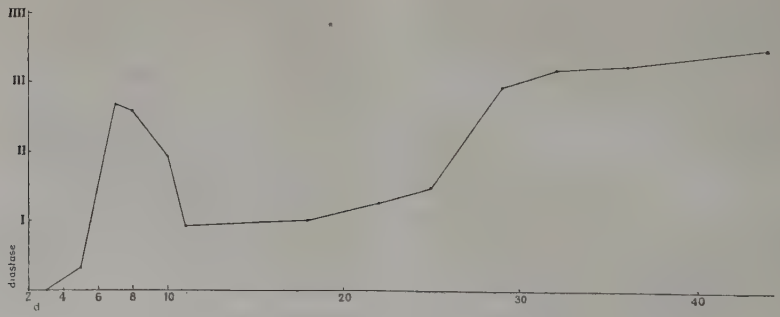

Fig. 9. Starch 2%. Diastase in the culture solution, (d = days).

The curve of the formation of diastase in this case is very similar to the curves, given for the series on 4 and 2.5% glucose. Development however was slow, so that the maximum dry weight was reached later. The curve reaches its minimum only on the 11th day.

Starch 0.5% (7—11; 155) fig. 10 (———), table 6 A.

A A i. e. inoculated with conidia from a culture on starch 0.5%.

Starch 0.5% (9—13; 177.5) fig. 10 (-------), table 6B.

G A i. e. inoculated with conidia from a culture on glucose 5%.

These two parallel series too show remarkable differences due to the conidia, with which they were inoculated, originating from different culture solutions. As also was the case with the homologous series on 5% glucose, we find a greater development of the dry weight of the mycelium in the series, where the conidia were derived from a different culture solution. At the same time less diastase was produced and in this series too a longer time elapsed before there was a homogenous and strong development of conidia. The differences are therefore analogous to those, found for the cultures on glucose, but they differ in degree.

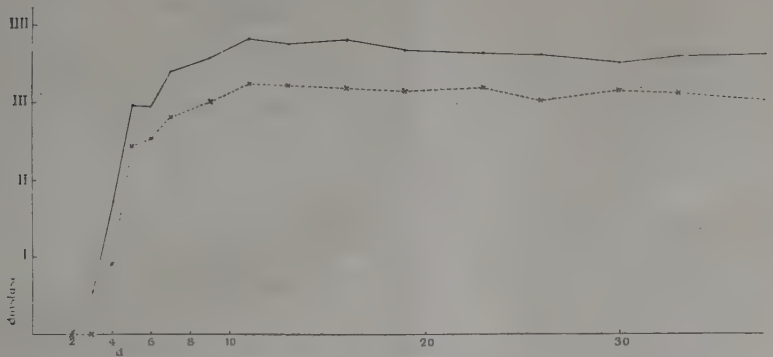

Fig. 10. Starch 0.5%. Diastase in the culture solution. A A———; G A--------- (d = days).

These curves remind us strongly of the curve for the glucose 1% series. They both show only a small serration, which indicates where there is a fall and subsequent rise takes place.

In a third series on 5% starch (fig. 11, table 6C) there was a rather large precipitate of starch after the culture solutions had been sterilised. This precipitate evidently could not be assimilated by the fungus, as is clearly shown i. g. by the dry weight of the 13th day: 116 m.Gr. Besides the mycelium did not form a con-

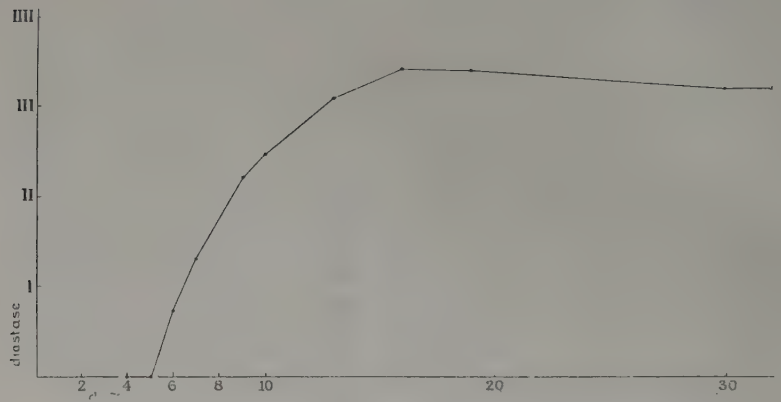

Fig. 11. Diastase in the culture solution. Starch 0.5%; (d = days).

tinuous pellicle but there were large openings in it. We may safely take this series to be one, in which less than 0.5% of foodmaterial has been given. The most remarkable fact about it is, that in the curve there is not even the slightest indication of a fall.

I intended to use this series for a special purpose, rather outside the range of the present subject; that is why I did not take all the usual observations. I am therefore not quite sure, that the dry weight on the 13th day (which was the only weight recorded, except that of the 32^{d} day on which it was 95 m.Gr.) is really the maximum weight. It is however highly improbable that it is far out. Although this series was inoculated with conidia from a culture on starch solution of 0.5%, from a culture solution of the same composition therefore, we find that the production of diastase is considerably weaker than that in series A A. The cause of this may be found in the scanty development of mycelium, but I can say nothing definite about this.

Starch 0.1% + Glucose 4% (9—13; 902) fig. 12 and 12 A, (------) table 7.

The curve for the production of diastase in the culture solution again shows the same characteristics as the curve of glucose 4% etc.

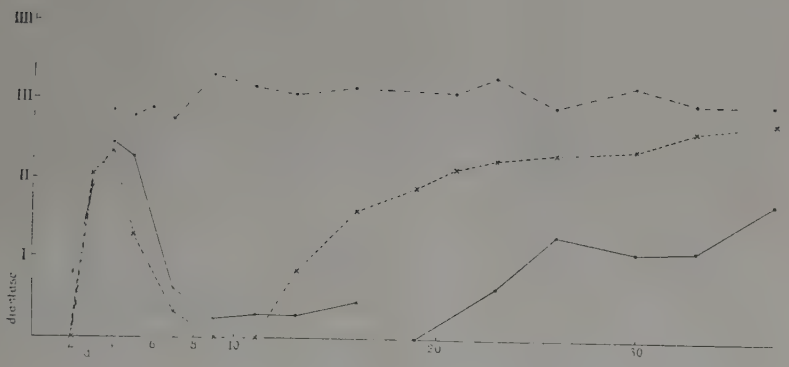

Fig. 12. Diastase in the culture solution.
Starch 0.1% + Glucose 4%: ———
Starch 0.25% + Glucose 2.5%: ·····
Starch 0.4% + Glucose 1%: —·—·— (d = days).

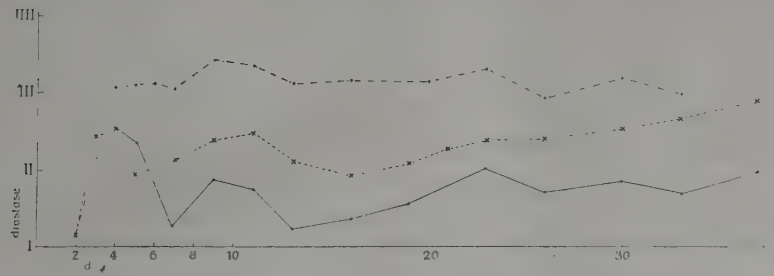

Fig. 12 A. Diastase in the mycelium + culture solution.
Starch 0.1% + Glucose 4%: ———
Starch 0.25% + Glucose 2.5%: ·····
Starch 0.4% + Glucose 1%: —·—·— (d = days).

The minimum is reached on the 9th and 19th days, but the rise after that is much smaller than in any of the cases, we have observed so far. Not much can be gathered from the curve of the enzyme production for the culture solution + mycelium (fig. 12 A ———); one can see indications of the usual course of events, but it is not very pronounced.

Starch 0.25% + Glucose 2.5%. (7—13, 636.5) fig. 12 and 12A, (--------) table 8.

The curve for diastase production in the culture solution (fig. 12 --------) does not show anything new. The minimum is again reached on the 9th day. The curve for the total production (fig. 12A --------) also shows little, at least on preliminary inspection; I shall return to this point later.

Starch 0.4% + Glucose 1%. (7—13; 357) fig. 12 and 12A (-·-·-), table 9.

The curves for this series are very similar to that for glucose 1%; as a matter of fact, both are almost identical.

B. Conclusions.

All the curves that I have discussed so far, make one fact clear at least, namely that for diastase formation it is immaterial whether starch or glucose is given in the culture solutions. The important changes and differences that have been observed, are not the result of the qualitative composition of the foodmaterials, but of the quantity supplied.

I believe that I am entitled to interpret my results as follows. Normally the production of diastase by the fungus rises to a maximum and after this maximum is reached, no more diastase is produced; but the quantity already present, retains its power and concentration for an indefinite time. This course of events is however only seen when nutrition is scanty i. e. less than 0.5% of foodmaterial is supplied in the culture solution. If the foodsupply is abundant, the mycelium secretes many substances into the culture solution. Amongst these there are some (proteids?) which adsorb the enzyme or inhibit its activity in some other way. This inhibition can already be seen during the first days of development, namely from the fact that only a low maximum is reached and after that the concentration even sinks for some time. This sinking of the concentration is rather inversely proportional to the dry weight and coincides more or less with the time, when the maximum

weight is reached, i. e. the stronger the development of the fungus, the more of these inhibiting substances are produced. As the development is dependent on the foodsupply, the latter must be considered to be the primary cause. After some time, these inhibitory substances are broken down or assimilated by the fungus, or removed in some other way. In any case, the diastase is again set free and now we find there appears to be a sudden rise. So for the first time, we are in a position to really estimate all the diastase that has been produced by *Aspergillus niger*. So much enzyme is set free in this way that the curve shows a second maximum, which is much higher than the first and remains almost at the same level for a long time[1]. We can also see now why the curves, which represent the production of diastase in the mycelium + culture solution (as for example in fig. 12A), follow a course from which very little can be learnt. The enzyme in the mycelium is not under the influence of the inhibitory substances in the solution and it therefore partially counteracts the fall which is seen, when the culture solution is observed alone; that is why it seems to disturb the course of the curve to some extent, when it is drawn to represent the amount of diastase, found in both the culture solution and the mycelium.

We might also imagine that the sugars, which are still present in the solutions, disturb the chemical equilibrium of the reaction and retard the hydrolysis in this way. This really takes place, when maltose is added to a mixture of diastase and starch solution. In an experiment I obtained the following results. I used a solution of diastase, which completely hydrolysed a given amount of starch in 185 secs. If the mixture contained 1% maltose, it took 440 secs.

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[1] Perhaps we may look upon the sudden rise of diastase concentration on the last day of observation of the series A G, table I B, as the beginning of this "setting free" of the enzyme. The dry weight in this series was very high: 1217 m.Gr., so that we may imagine that so much of the inhibitory substances was secreted, that almost all the diastase had been neutralised, but that in the end the setting free began, so that on that last day a rather large amount of diastase was found. This single observation however, does not suffice to entitle us to this conclusion.

and with 2% maltose 1140 secs. As the enzyme of *Aspergillus niger* changes starch into maltose (see page 251, note), the experiments on glucose are not influenced by this factor at all, but where the culture solution also contains starch, the maltose, which is formed, may act as an inhibitory influence during the first few days. This can not be the main cause however, because the minimum is only reached some time after all of the foodmaterials have been consumed.

The total amount of enzyme, produced at the end of the experiment, is always the same, no matter what the concentration was of the culture solution. In the series on 0.5—2.5% at least, we see that the same high maximum is reached in all cases. The maximum is not so high in the series on 4%, but in this case the inhibitory substances are formed more abundantly and it will therefore take a longer time before all of the diastase has again been set free. The same of course is true of the 5% solution.

I therefore draw the conclusion, that neither qualitative nor quantitative changes in the foodmaterial have any influence at all on the amount of diastase which is produced by *Aspergillus niger*. This must be true of culture solutions containing glucose and (or) starch at least. A high concentration temporarily seems to change the course of events. That is why I think that the curve in fig. 11, for the third series on starch 0.5%, is the only one, which gives a true picture of the course, taken by the production of diastase of the fungus (compare also fig. 2).

Some important facts seem to support the view I hold on this matter.

Turning to fig. 13 and table 10 on the series, grown on 5% maltose (11—13; 1040.5), we see that there is an apparent maximum, which is followed by a decrease. This fall in concentration is however not nearly as large as in the case of the 5% glucose series, and the subsequent rise is not so great either. Evidently *Aspergillus niger* produces very little maltase. If a culture solution, containing maltose is used, the maltose takes a very long time to disappear. Glucose and other foodmaterials had generally disappeared on the

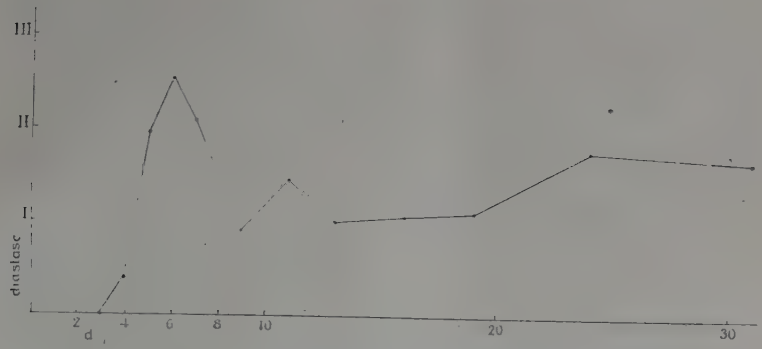

Fig. 13. Maltose 5%. Diastase in the culture solution, (d = days).

10th or 12th day, but in this case a considerable quantity of sugar is left even on the 24th and 31st day[1], as can be seen from the rotation of the culture solution in the polarimeter. This is in other series zero on the days in which the maximum dry weight is reached, i. e. the 10th or 12th days; here it is = 1°23′24″ on the 24th day, on which the dry weight only has diminished to 911 m.Gr. Dissimilation has here gained the upperhand, yet assimilation is still so active, that there is little decrease. So it is probable, that at the same time substances are secreted, which prevent the diastase from unfolding its full powers. The maltose itself must also retard the action of the enzyme. If the series had been longer proceeded

[1] The rotation of the culture solution shows, as might have been expected, that *Aspergillus niger* changes maltose into glucose. This however takes such a very long time, that we can not imagine glucose is the end product of the hydrolysis of starch. This hydrolysis in most cases is so rapid, that the end product can not be but maltose.

I am strengthened in this opinion by the following fact: in the third series, grown on 5% starch, the reducing power of the culture solution was tested every day. When on the 9th day all the starch had disappeared, the reducing power of the solution was found to be equal to that of a 0.4% maltose solution i. e. equal volumes of 0.4% maltose solution and culture solution reduced the same amount of Fehlings solution. This is not a positive proof that in this case maltose is the end product, but it makes it very probable.

with, I feel sure that at a later date, just as much amylase would have been found as in all the other series. Unfortunately I had not enough cultures and did not have the time to grow them.

A large number of facts, which I got from totally different observations seem to confirm this view; or at least they can be explained by it in a very reasonable way.

I have already mentioned that after, had done the above observations, I saved what was left of all the culture solutions. Daily or weekly tests were regularly performed to see, whether their concentration of diastase was diminishing and if so, how. It is hardly possible to give all my results, as there are some hundreds of observations. There is also no necessity to do so, because I can state in a few words what can be learnt from them.

The culture solutions of the three series, grown on 0.5% starch, seemed to preserve their enzyme concentration for an indefinite time. The hydrolysing power of these solutions was as great after about ten months as it had been on the first day when the solution had been filtered from the mycelium[1]. There are a few exceptions to this however in the case of the A G series. The reader will remember that the development of mycelium was the greatest in this series of the three and reached its maximum between the 9th and 13th days. The culture solutions which had been obtained on these four days showed a decrease in the enzyme concentration. After 34 days there was found to be left of the original concentration:

in the liquid of the	7th day	50 %
	9th „	60 %
	11th „	78 %
	13th „	82 %

So there were enough of the inhibitory substances in these solutions to retard the action of the enzyme.

Of the A A series I allowed one culture to grow for 175 days and then I proceeded with it in the usual way. I expected that the

[1] This makes clear that the influence of the electric light during the hours of the experiments, has been none.

products of decomposition by the mycelium would have completely destroyed or inhibited the action of the diastase. Yet the enzyme concentration was found to be = 577, i. e. rather considerable and after about seven months, 72% of the original concentration was still found. The dry weight of this 175th day was 110 m.Gr. i. e. hardly less than the weight on the 40th.—50th days of this series. There can therefore be no reason to assume, that there were strong processes of decomposition and this explains why such a relatively large amount of diastase was found.

In the case of the series on 2% starch, I could only save the liquids of the last four days of observation, viz. of the 29th, 32nd, 36th and 45th days. As the curve for enzyme concentration shows the rise long before this, I assumed that most of the enzyme was free by this time and I expected that the liquids would loose little of their hydrolytic powers. This expectation was confirmed.

the culture solution of the	29th	day, after 62	days	still contained	82%
	32th	„ „ 124	„	„	100%
	36th	„ „ 131	„	„	90%
	36th	„ „ 233	„	„	75%
	45th	„ „ 131	„	„	79%

It is quite clear too what we may expect from the series on 1—2.5—4 and 5% glucose, viz. the enzyme concentration should be constant in most of the 1% culture solutions. Only those of the days of maximal growth could possibly show a gradual decrease of diastase concentration. The higher the concentration of the glucose was, the more of these liquids would not remain constant and the more rapidly they will loose their strength.

These expectations were realised. In the case of the 1% glucose series (9—16; 261) the culture solutions of the 5th till the 9th days were not constant. After 12 days the following amounts were left:

in the solution of the	5th	day	69%
	6th	„	70%
	7th	„	50%
	9th	„	62.5%

For the 2.5% series (11—13, 615.5) the same holds good for the 5th—18th day; 12 days after filtering, the following amounts were left:

in the solution of the	5th	day 70%
	6th	„ 86%
	7th	„ 61%
	11th	„ 8%
	13th	„ 16%
	16th	„ 9%
	18th	„ 62%

One can already see the influence of the increasing dry weight. This is also the case for the series on 4% glucose. After 10 days the following amounts of enzyme were left in the liquids obtained from the cultures between the 5th and the 18th days:

in the solution of the	5th	day 56%
	6th	„ 46%
	7th	„ 33%
	11th	„ 16%
	13th	„ 7.8%
	16th	„ 86%
	18th	„ 50%

In this series also the liquids from later days were not quite constant. In most cases 20 to 30% of the diastase had been lost after about two months.

In the case of the 5% glucose series (11—19; 944.5), only the solutions of the last few days have been kept. From the curve in fig. 4 it can be seen, that all the enzyme is never set free. It is therefore quite in accordance with this fact, that even these solutions should loose their strength very rapidly. After 6 days they retained only the following amounts of their enzyme:

the solution of the	33th	day 8%
	37th	„ 9%
	40th	„ 50%
	46th	„ 10%

We may therefore safely assume, that none of the liquids of this series would have kept their strength for any length of time.

I could not observe all my solutions so long as I should have wished to, because in many cases my supply gave in within a very short time. It was therefore impossible to give observations which had been made after an equal length of time in all cases. I could of course make the number of days, on which the observations were taken, the same for all series, but then I should have to exclude all the results, which I obtained after the smallest number of days, during which those observations were made i. e. 6 days (in the case of the 5% glucose series). Besides, the results would have shown nothing. Yet as they are, they show very clearly the influence of the increase in fungous matter, proving that this influence is directly proportional to the decrease of enzyme action.

The series on maltose gave very poor and disconnected results here.

In the case of 0.1% starch + 4% glucose (9—13; 902), there was no decrease in the liquids of the 3^{d} and the 4th days. No further observations were made.

In the liquids from the 0.25% starch + 2.5% glucose series (7—13; 636.5) the following amounts were left after 6 days:

in the solution of the 4th day 30%
18th „ 24%
19th „ 20%
21th „ 21%
23th „ 12%

Liquids from latter days did not loose more than about 15%.

After standing for 12 days, the liquids from the 0.4% starch + 1% glucose series (7—13; 357) gave the following amounts:

in the solution of the 4th day 54%
5th „ 7.5%
9th „ 2.5%
11th „ 11%
13th „ 13%

The influence of the inhibiting factors in this case is greater than I expected to see, when considering the relatively small dry weight of the mycelium.

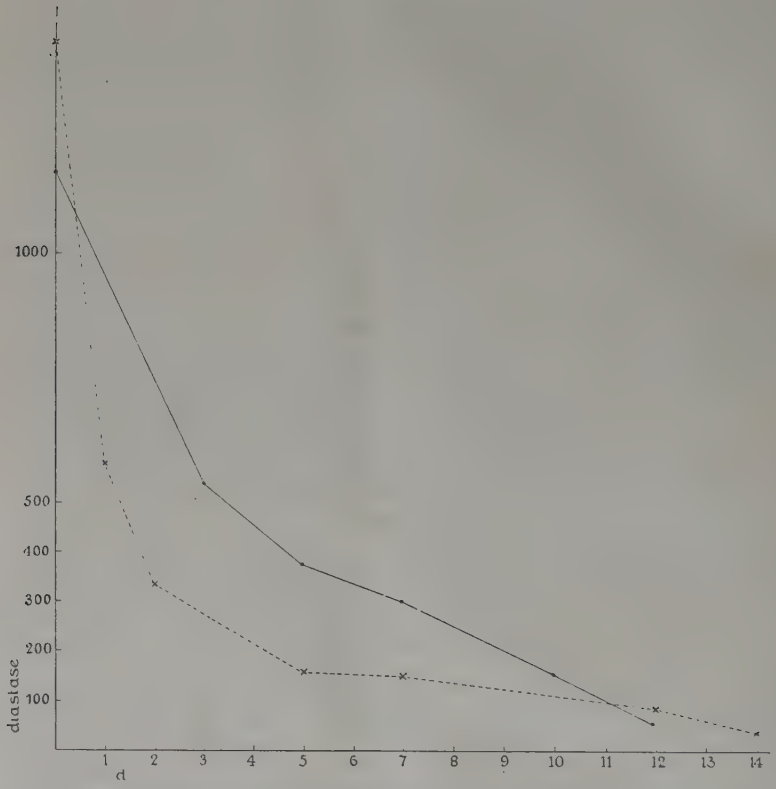

Fig. 14. Decrease in enzyme concentration in the culture solution; series on 0.4% starch + 1% glucose; 11th ·-·-·-·- and 13th day ——— (d = days).

Fig. 14 represents the decrease of the enzyme concentration in the culture solutions of the 11th and the 13th days of the last mentioned series. These curves make it very clear, that the inhibition of the diastase takes place according to the law of mass-action. That is why it is not desirable to compare the results of a slow inhibition with one, taking place very quickly, by making observations on both after an equal and small number of days.

It is quite conceivable that these inhibiting substances may in many cases be present in totally different quantities. Granting

this, we are not surprised that the results, described above, show so many irregularities. More over tables 7—9 show, that individual differences between the diastase concentrations of two cultures, examined on the same day, are greatest on those days, where we should expect the strongest secretion of inhibitory substances.

Just at present, I am as yet unable to give the quantitative relations between the mass of inhibiting substance and the mass of enzyme that is bound by it. I have attended to do by salting out, by precipitation and by other methods, but without result. I can only state positively that we have to deal with very small quantities here. The liquids containing diastase were perfectly clear without exception. $(NH_4)_2SO_4$ even in high concentration gave no perceptible sediment.

IV. SERIES ON SACCHAROSE, GLYCERINE AND LACTOSE.

The series under discussion now, were rather unsuccessful, as far as diastase production is concerned. Yet they show some very remarkable points.

Four series were grown on a solution containing 5% saccharose, viz.

one inoculated with conidia from a culture on glucose 5%.
„ „ „ „ „ „ „ „ saccharose 5%

and two similar parallel series, but to which were added small quantities of $ZnSO_4$ and $FeSO_4$ (0.0025%; Raulin). This was done because I wanted to try, if the wellknown influence of these salts on the dry weight, would also influence the production of diastase.

All these series formed heavy mycelia, which showed large undulations on their surfaces. The cultures to which iron and zinc salts had been added showed a rather dark lemon colour, due to the iron. The mycelia in these series were very brittle and could not be removed from the flasks in a single piece as they broke easily. Formation of conidia in these also was scanty and only along the sides. The cultures which did not contain zinc and iron

were covered after some time by a layer of conidia, which was not very dense.

Saccharose 5%. (28—37; 1058), table 11 A.
G S: i. e. inoculated with conidia from a culture on 5% glucose.
Saccharose 5% (18—22; 989), table 11 B.
S S: i. e. inoculated with conidia from a culture on 5% saccharose.

Development of diastase in the culture solutions of both series is very insignificant. After the 13th day a small amount of diastase was found in the mycelium of G S and this for all further observations remains practically constant. In S S not even this small amount of diastase is found. Such small quantities are formed, that they may safely be neglected. The hydrolysis of the saccharose and the assimilation of the inverted sugar almost took equal times in both series. I have no explanation to offer for the fact, that the maximum dry weight in G S was reached later than in S S.

Saccharose 5% + Fe + Zn (11—16; 1426) table 12 A.
G S′: i. e. inoculated with conidia from a culture on 5% glucose.
Saccharose 5% + Fe + Zn (11—18; 1417) table 12 B.
S S′: i. e. inoculated with conidia from a culture on 5% saccharose.

The dry weight here is really greater than in the former series to which no zinc and iron salts had been added; besides the maximum weight is reached much sooner.

After the 11th day some diastase appears in the mycelium of G S′ which on the 26th day has disappeared again. This is about double the amount found in the G S series above and this relative increase is probably due to the influence of zinc and iron ions. The fact that in S S′ there is no diastase formation, as it was the case in S S, is the only indication of the differences, due to the origin of the conidia. Nevertheless a simple fact like this one may cause two observers to disagree entirely, simply because they did not notice from what type of culture they obtained the conidia, from which they grew their cultures.

The most striking fact in these observations is the small amount of diastase formed on saccharose. As far as I know, no investigators before me found such large differences of diastase production on starch, glucose and saccharose. Katz and Kylin always speak of "reichliche Mengen", but this may be so, because they always mixed their saccharose with 0.25% starch. Most other investigators give equally vague indications, and we may draw the conclusion that they never found much less diastase production on saccharose than on other sugars. It is possible that in my case the large dry weight of the mycelium may have influenced the results. We can safely imagine this to be the case for the series on cane sugar + $Fe^{\cdot\cdot}$ + Zn.. Here the weight amounted to 1426 and 1417 mgrs. and perhaps we may add to this a possible disadvantageous influence of the iron and zinc. In the S S and G S series it is much less probable that we should consider the influence of the dry weight (989 and 1059 mgrs.). In the first case it only differs very slightly from that of the series G G (944 mgrs.) where there was a considerable development of diastase. In any case it is conceivable that this small increase in dry weight just may be able to turn the scale.

If all this is true, we might expect that *Aspergillus niger* should produce large quantities of diastase when cultivated on lower concentrations of cane sugar, e. g. 1%. I tried to find out if this really was the case. For this purpose I made six cultures on 1% saccharose (with conidia from a 4% glucose culture). Two of these I examined on the 7th, 14th and 21st days. The results may be seen in the following table:

days	diastase in the myc.	P_H of the myc. extract	diastase in the culture solution	P_H of the cult. sol.	dry weight
7	25	6.5	18.7	6.65	54
14	23	3.05	21	2.95	274
21	50	2.65	524[1]	2.50	303

[1] This result is the average of the observations on two cultures.

Development was very slow in the beginning. On the 7th day the results as a whole were negative; on the 14th day the degree of acidity and development of mycelium were such, that a good production of amylase could have been expected; yet none was found. So we assume that not only the concentration, but also the chemical constitution ot the cane sugar made itself felt. The amounts of diastase that did appear, were very small and may be attributed to the influence of the 4% glucose solution on which the conidia, I started off with, were grown.

On the 21st day however, the results were entirely as I had expected them to be. The quantity of diastase found in the culture solution was, it is true, not quite as great as on 1% glucose; yet there was such a great rise in diastase production, that I do not hesitate to accept this as an absolute confirmation of my supposition mentioned above.

Michaelis and Menten have determined what influence admixtures have when they are added to the combination enzyme-substratum i. c. invertase-saccharose. They found that there are substances, which greatly diminish the affinity of the enzyme to its substratum. The split products of cane sugar did so to some degree, glycerine for instance had a much stronger influence. Perhaps we must consider saccharose and fructose as such admixtures in the starch-diastase combination. This fact would also explain why there was no diastase production on 5% saccharose: this experiment was not continued long enough to exclude all inhibitory factors, first the saccharose and fructose, later on the great weight of the fungus. If once we succeed to overcome these sources of errors, the production of diastase can be observed as happened in the small experiment on the 1% saccharose cultures.

Glycerine 5% (28—33; 1021) table 13 A
inoculated with conidia from a culture on 5% glucose;
Glycerine 5% (29; 998) table 13 B
inoculated with conidia from a culture on 5% glycerine.

Development on glycerine was very slow; in the end however

there appeared compact, rolled up and detached bits of mycelium, which floated on the surface of the liquid. They were light grey because of the conidia, which were closely attached to them.

As can be seen from both tables, there is no production of diastase in either of the series worth while mentioning. But we may not conclude from this, that *Aspergillus niger* produces no diastase on glycerine. We ought to find out first, if we can obtain the same results with lower concentrations of glycerine, as we got with 1% cane sugar. At present I can only state, that in cultures an 5% glycerine, the factors which possibly inhibit the production of diastase, have not yet disappeared after 52 days.

Two parallel series were also grown on 5% lactose. On this culture solution there was almost no development. There seems to be no lactase in *Aspergillus niger* and consequently it can not be produced, even if it is necessary. The rotation of the culture solution was $2^{\circ}28'3''$ on the first day and $2^{\circ}24'36''$ on the 19th day. There were very small bits of pellicle formed, which were so scanty, that they only became visible, when coloured by the development of conidia. As these results are of no importance, I have not tabulated them; it is almost superfluous to mention, that no amylase was produced.

V. SUMMARY.

Before we experiment with the enzymes of fungi, it is imperative that we should know, what hydrogen ion concentration is wanted for the optimal reaction of these enzymes. *Aspergillus niger* produces the necessary degree of acidity in its own culture solution. This however is not the case with all fungi.

It is of the utmost importance to know, what nutrient base is used in the cultures, from which we obtain the conidia for our experiments. It is always best to take these conidia from cultures on solutions of the same chemical composition as those, on which we intend to grow them.

Aspergillus niger produces large quantities of diastase from the beginning of its development until a maximum is reached. Then

no more is secreted, but this maximum amount, which is then present, remains constant during the entire lifetime of the fungus. This was found to be the case when cultivated on glucose, starch and maltose. Neither the chemical composition, nor the quantity of these substances seemed to have any influence.

If the concentration of the culture solution is higher than 1%, it may disturb the course of enzyme production temporarily, because certain substances are formed, which inhibit the action of the enzyme. These substances disappear during the course of development and the normal maximal quantity of enzyme is eventually produced.

The diastase of *Aspergillus niger* is secreted into the culture solution immediately after it is formed.

The diastase may be kept for a long time without loosing its strength.

Saccharose has a deleterious influence on the action of diastase. For some reason or other, the normal amount of diastase appears, after all the cane sugar and its split products have disappeared from the culture solution.

When *Aspergillus niger* was grown on 5% glycerine, no diastase production could be proved at all.

Aspergillus niger has no lactase, even if it is grown on lactose. There is therefore practically no development of the fungus on this substance.

Before I close I have pleasure to thank prof. Dr. F. A. F. C. Went, not only for his very valuable help, but also for his kindly allowing me to proceed with his own experiment and for putting at my disposal all the facilities of the Botanical Laboratory in Utrecht. Without this very material help, I should have been unable to carry out this research. I therefore wish to express my heartfelt thanks at this point.

Utrecht, Botanical Laboratory 1920—1922. Presented April 1922.

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VII. TABLES.

Table 1 A. G G glucose 5%. | Table 1 B. A G glucose 5%.

days	diastase in the mycelium[1]	diastase in the culture solution[1]	dry weight in m.Gr.	diastase in the mycelium[1]	diastase in the culture solution[1]	dry weight in m.Gr.
2	2.7	8	71	3.5	4.5	67
3	2.5	10.5	92	3.8	4	112
4	75.1	190	315	11.5	12.5	264
5	40	300	357	8.5	13.5	399
6	35	180	524	6	5.5	571
7	15	20	615	3.5	0	885
9	16.5	0.75	818	15	0	1330
11	23	0	981	2.5	0.35	1125
13	10	0.9	993	9	0	1213
16	30	0	865	12.5	0	1200
19	50	2.5	939	8.1	0	956
23	68	12.3	714	6	0	886
26	10	20.9	724	7.5	1	851
30	33	31.5	671	9.4	2.9	889
33	9.1	92.5	656	8.6	2.3	841
37	60	454.5	—	—	—	—
40	25	364	698	10.7	16.4	779
46	17		671	11.7	160.	807

[1] The figures in these columns are the averages of the results of observations on at least two cultures.

Table 2. Glucose 4%.

days	rotation of the culture solution 10 c.M.	diastase in the mycelium	PH of the mycelium extract	diastase in the culture solution	PH of the culture solution	dry weight in m.Gr.
3	1°47′24″	7.5	5.8	10	5.9	—
4	1°40′48″	10	5.25	33.3	3.8	112
5	—	68	4.25	70	3.6	291
6	—	—	—	75.5	2.6	510
7	1°18′ 0″	—	—	65.2	2.3	505
9	—	—	—	18.7	2.0	643
11	0°37′48″	—	—	93.5	2.0	864
13	0°24′ 0″	—	—	150	2.0	922
16	0°11′24″	—	—	2500	1.95	899
18	0° 9′ 0″	375	3.1	2308.2	2.1	819
23	—	—		2000	4.2	763
26	—	428	3.2	3000	4.3	696
31	—	37.5	3.2	750	4.4	683
37	—	150	3.3	2500	4.2	580

Table 3. Glucose 2.5%.

days	rotation of the culture solution	diastase in the mycelium. water-extract	PH of the water-extract of the mycelium	diastase in the mycelium extract of the culture solution	PH of the mycelium. extracted with the culture solution	diastase in the culture solution	PH of the culture solution	dry weight in m.Gr.
3	1° 5′24″			7.5	5.4	10.7	5.9	—
4	1° 0′ 0″	12.5	6.9	28	5.2	33.3	4.6	54
5	—	10.7	6.6	75	3.6	83.3	3.9	117
6	—	10	6.2	111	3.4	125	3.2	351
7	0°43′12″	13.7	6.25	—	—	176.5	3.—	359
9	—	14.4	6.05	100	4.2	68.2	2.05	435
11	0° 4′12″	22.4	6.0	—	—	130.4	2.—	617
13	0° 0′14″	16.7	6.1	—	—	167	1.95	614
16	0° 0′ 0″	82.5	5.9	—	—	714.3	2.1	542
18	—	30	6.1	50	3.2	2308.2	2.9	543
23	—	12	6.0	88.2	3.25	1071	3.2	481
25	—	57.7	6.2	250	3.4	1154	3.25	—
26	—	—	—	428.7	3.2	1875	3.2	434
31	—	30	6.15	166.7	3.3	2308.4	3.2	472

Table 4. Glucose 1%.

days	rotation of the culture solution 10 c.M.	diastase in the mycelium	P_H of the mycelium extract	diastase in the culture solution	P_H of the culture solution	dry weight in m.Gr.
3	0°25′12″	27.8	5.45	5	6.6	38
4	—	166.7	5.1	200	3.55	107
5	0°12′ 0″	349	3.45	789.5	3.05	161
6	—	357	3.25	833.3	3.—	299
7	—	—	—	1071.4	3.—	234
9	—	—	—	2000	2.9	251
11	0° 0′ 7″	—	—	3000	3.—	275
13	0° 0′ 0′	—	—	2727.3	3.—	263
16	—	—	—	5454.5	3.05	254
18	—	—	—	6000	3.15	229
23	—	—	—	4286	3.55	238
26	—	—	—	4286	3.4	224
31	—	84	3.3	6000	3.5	254
37	—	18.5	3.4	6000	3.35	205

Table 5. Amylum 2%.

days	diastase in the mycelium	P_H of the mycelium extract	diastase in the culture solution	P_H of the culture solution	dry weight in m.Gr.
3	4.6	7	0	6.5	135
5	0	4.8	2	5.1	201
7	6.4	4.6	457.1	3.4	248
8	10	4.65	333.3	3.1	
10	16.6	4.2	83.3	3.15	
11×	12.5	4.—	8.2	2.4	499
18	9.7	4.—	10	2.05	486
22	5.4	3.95	18.7	2.4	495
25	5	3.9	30	2.1	417
29	3	4.1	833.3	3.1	380
32	3.3	4.0	1500	3.9	
36	3.5	4.2	1666.7	3.8	372
45	3.75	4.35	3000	3.85	

days	Table 6 A. Amylum 0.5% A A. diastase in the mycelium[1]	diastase in the culture solution[1]	dry weight in m.Gr.	6 B. Amylum 0.5% G A. diastase in the mycelium[1]	diastase in the culture solution[1]	dry weight in m.Gr.	6C. Amylum 0.5%. diastase in the culture solution	PH of the culture solution
2	0	0	1	0	0	4	—	—
3	2	3.5	26	3.75	0	9	—	—
4	300	53.5	71	250	79	103	—	—
5×A	280	900	122	120	270	121	0	7
6×B	560	900	122	220	320	104	5.4	6.6
7	140	2500	152	210	790	158	20	6.1
9×C	33	3390	160	130	1000	191	166.7	4.6
10	—	—	—	—	—	—	300	4.05
11	20	6330	152	37.5	1760	172	357.1	3.8
13	12	5290	144	15	1680	170	1250	3.35
16	10	6260	129	25	1550	144	2500	3.1
19	8.6	4670	110	12.5	1450	139	2307.7	3.6
23	67.2	3960	111	30	1560	111	—	—
26	17.8	4440	133	93	1077	137	—	—
30	9.2	3780	141	23.6	1560	140	—	—
32	—	—	—	—	—	—	1579	3.9
33	15	4090	130	13.6	1410	132	—	—
40	104.2	4749	140	95.2	993.6	146	—	—
46	14.6	5292	122	13.6	672.5	110	—	—
52	10	4398	—	12	638.5	139	—	—
58	—	—	—	21.4	675.3	147	—	—

[1] The figures in these columns are the averages of the results of observations on at least two cultures.

Table 7. Amylum 0.1% + Glucose 4%.

days	diastase in the mycelium, water extract	diastase in the mycelium, extracted with the culture solution	diastase in the culture solution of flask 1	diastase in the culture solution of flask 2	average of flask 1 and 2	dry weight in m.Gr.
2	8	7.5	6	7	6.5	91
3×	9.4	53.6	50	120	85	174
4×	30	53.6	214.5	333.3	273.9	390
5	33.3	42.8	33.3	333.3	183.3	403
7	15	15	4.2	4.2	4.2	775
9	115	75	3	0.6	1.8	913
11	50	55	1.5	2.5	2	908
13	67	16.6	1	3	2	885
16	48	20	1.5	4.2	2.9	775
19	42.9	37.5	1	1	1	793
23	33.3	100	4	4.5	4.3	621
26	23	33.3	20	—	20	—
30	130	60	8.8	16.4	12.6	649
33	53.6	37.5	11.5	15	13.3	—
37	6.8	44.1	78.9	30	54.5	576

Table 8. Amylum 0.25 % + Glucose 2.5 %.

days	diastase in the mycelium, water-extract	diastase in the mycelium, extracted with the culture solution	diastase in the culture solution of flask 1	diastase in the culture solution of flask 2	average of flask 1 and 2	dry weight in m.Gr.
2	4.9	15	0	0	0	56
3	21.4	158	130	88.2	109.1	152
4×	33.3	120	166.7	250	208.3	372
5	33.3	68.1	15	25	20	477
7	54.2	143	3	2.5	2.75	680
9	166.7	250	2	3	2.5	649
11	50	300	4.3	2	6.1	613
13	75	120	7.5	7.1	7.2	604
16	39.5	44.1	41.8	41.8	41.8	454
18	25	75	50	10.7	78	465
21	28.8	50	142.8	—	142.8	—
23	28.3	62.5	200.5	166.7	183.3	360
26	2.5	43	214.5	—	214.5	—
30	3.4	85.7	250	250	250	447
33	7.5	40	428.6	—	428.6	—
37	4.3	63.8	428.6	680.9	554.6	403

Table 9. Amylum 0.4% + Glucose 1%.

days	diastase in the mycelium, water-extract	diastase in the mycelium, extracted with the culture solution	diastase in the culture solution of flask 1	diastase in the culture solution of flask 2	average of flask 1 and 2	dry weight in m.Gr.
2	3.5	15	0	0	0	47
3	5	58.2	107	65.2	86.1	93
4×	30	454.6	652	750	701	237
5	50	600	441	750	595.5	319
6	43.3	555.5	750	—	750	—
7	47	555.5	375	714	544.5	378
9	176.4	625	1875	2145	2010	347
11	120	750	1500	1364	1432	355
13	45.4	200	1150	1150	1150	349
16	17.3	49	1250	1666.7	1458	295
19	39.5	187.5	1580	1111	1346	278
21	32	57.7	1250	—	1250	—
23	1	60	1500	2500	2000	292
26	3.9	34	833.3	—	833.3	—
30	11.1	39.5	1875	1363	1614	279
33	4.3	40	967.7	—	967.7	—
37	7.4	—	1000	935	976.5	307

Table 10. Maltose 5%.

days	rotation of the culture solution 10 c.M.	diastase in the mycelium	P_H of the mycelium extract	diastase in the culture solution	P_H of the culture solution	dry weight in m.Gr.
3	5°29′ 6″	0.5	7	0.4	6.43	—
4	5°41′ 6″	4.4	6.83	2.5	6.3	—
5	5° 9′ 0″	0	4.5	93.5	4.5	240
6	4°38′12″	0.4	3.1	333.3	2.75	410
7	—	25	2.9	157.9	2.06	580
9	2°53′31″	36.3	2.25	8.3	1.85	856
11	2° 3′36″	37.5	2.1	30	1.80	1041
13	2°10′24″	11.5	2.—	10	1.80	1040
16	1°45′34″	18.7	1.9	15	1.95	996
19	1°33′36″	18.7	2.1	16.7	1.90	924
24	1°23′24″	39.5	2.05	57.8	1.95	911
31	0°43′21″	—	—	50	2.—	853

Table 11A. Saccharose 5 %. GS.

days	rotation of the culture solution 10 c.M.	diastase in the mycelium	PH of the mycelium extract	diastase in the culture solution	PH of the culture solution	dry weight in m.Gr.
2	+3°45′20″	—	—	18.7	7	1
3	+3°45′ 3″	7	7	11.3	6.2	120
4	+1°32′14″	8.8	6.8	12.9	6.0	179
5	+0° 6′ 5″	1.4	6.1	1.3	5.2	248
6	−0°38′22″	0.5	5.9	4.7	4.1	376
7	−1°43′12″	1.4	5.1	5	4.0	394
9	−1°29′36″	0	4.2	0	2.8	613
11	−1°21′52″	2.1	3.9	1.5	3.0	614
13	−1°19′41″	21.5	3.3	0	2.6	587
14	—	25	3.1	0	2.1	—
16	−1°12′24″	11.5	2.6	0	1.95	746
17	—	27.3	2.3	0	1.8	—
18	−1°43′12″	29.5	2.7	0	1.85	—
19	−1°10′28″	21.5	2.2	0	1.90	—
20	—	27.3	2.0	0	2.1	817
21	−1° 0′19″	34	2.4	0	2.2	—
24	−0°33′ 8″	25	2.6	0	2.1	886
26	0° 0′ 0″	28.7	2.6	0	2.4	—
28	—	15	2.7	0	2.7	1100
30	—	18.7	3.1	0	2.2	—
33	—	23	2.8	0	2.15	1040
37	—	25	2.9	0.5	2.4	1034

Table 11B. Saccharose 5 %. SS.

rotation of the culture solution 10 c.M.	diastase in the mycelium	diastase in the culture solution	dry weight in m.Gr.
+3°15′35″	0	4.5	—
—	—	—	—
—	—	—	—
+0°43′20″	0	1.5	148
—	—	—	—
—	1	2.5	—
—	—	—	—
−1°38′42″	1.2	1	688
—	—	—	—
—	—	—	—
—	—	—	—
—	—	—	—
−1° 0′36″	7.5	0	916
—	—	—	—
—	—	—	—
−0°32′22″	18	0	1062
—	—	—	—
0° 0′ 0″	7.5	0	901
—	7.1	0	850
—	3.7	0	—
—	—	—	—
—	15	0	903

days	Table 12 A. Saccharose 5% +Fe̤ +Zn̤ GS′ rotation of the culture solution 10 c.M.	diastase in the mycelium	PH of the mycelium extract	diastase in the culture solution	PH of the culture solution	dry weight in m.Gr.	12B. Saccharose 5% +Fe̤ +Zn̤ SS′ rotation of the culture solution 10 c.M.	diastase in the mycelium	diastase in the culture solution	dry weight in m.Gr.
3	+2°45′17″	6.5	6.8	12.5	6.8	118	+2°48′44″	1.8	0.5	—
4	—	9.5	5.9	9.8	5.8	182	—	—	—	—
5	+0° 0′22″	7.4	5.1	6.9	4.95	361	+0°32′22″	0	1	183
6	—	5	4.2	3.1	3.6	555	—	—	—	—
7	—	5	3.4	1.1	3.1	752	−0°16′24″	4.6	8.1	—
9	—	2.5	3.05	0	2.2	1115	—	—	—	—
11	−1°50′46″	46.8	2.45	0	2.1	1440	−0°32′24″	15	0	1464
13	—	23.1	2.0	0	1.85	1370	—	—	—	—
14	—	32	1.95	0	1.8	—	—	—	—	—
16	—	42.8	1.8	0	1.95	1469	—	—	—	—
17	—	45.5	1.85	0	1.8	—	—	—	—	—
18	−1°12′28″	26	2.1	0	1.90	—	0° 0′ 0″	9.6	0	1389
19	—	45.5	2.0	0	2.05	—	—	—	—	—
20	—	50	2.15	0	1.95	1203	—	—	—	—
21	−0°32′10″	42.8	2.2	0	2.1	—	—	7.5	1.2	1177
24	0° 0′ 0″	21.4	2.4	1.5	2.2	1194	—	12	4.4	1120
28	—	10	2.1	0	2.3	1057	—	6.9	2.9	1099
33	—	10.7	2.6	0	2.05	1042	—	3.3	21.4	—
37	—	9.5	2.6	1.4	2.3	1034	—	5	0	1103
44	—	5	2.55	11.5	2.2	—	—	—	—	—

days	Table 13A. Glycerine 5%. diastase in the mycelium	PH of the mycelium extract	diastase in the culture solution	PH of the culture solution	dry weight in m.Gr.	13B. Glycerine 5%. diastase in the mycelium	diastase in the culture solution	dry weight in m.Gr.
2	—	—	11	6.6	1	—	—	—
3	—	—	8.2	6.1	23	0	0	—
4	—	—	9	6.0	111	—	—	—
5	1.6	6.8	1.6	5.8	97	—	0	48
6	0.5	6.1	0.5	5.45	128	—	—	—
7	1.2	5.95	2.4	5.1	127	0	0	—
9	0	5.8	0	4.8	140	—	—	—
11	0	5.3	0	4.8	176	0.2	0.7	134
13	0	5.0	0	4.7	167	—	—	—
16	5	4.7	5	4.3	476	—	—	—
18	4.9	4.2	1.1	3.8	—	1.7	4	676
19	4.3	3.9	4.3	3.1	—	—	—	—
20	10	3.1	1.4	2.75	610	—	—	—
21	4.7	2.4	6	2.46	—	5	0	685
24	3	2.35	0	2.05	572	4.7	0	504
28	4.7	2.2	1.4	1.90	1052	4.4	0	998
30	13.6	1.95	0	2.05		—	—	—
33	5	2.3	1.3	1.95	990	7.5	0	—
37	5	2.1	0	2.2	943	7.5	0	882
44	2.5	2.4	0	2.72	—	—	—	—
52	5.4	2.7	0	2.35	—	—	—	—